Claims

1. An assay method for the determination of transcobalamin II bound cobalamin in a body sample, comprising contacting a cell free sample of a body fluid with an immobilised or immobilizable specific binding ligand for TC II or holo TC II, separating a ligand bound fraction from a non-ligand bound fraction and measuring the holo-TC II or TC-II bound cobalamin content therein.

2. An assay method as claimed in claim 1 wherein said specific binding ligands for TC II or holo-TCII allow for separation and concentration of the TC II or holo-TC II in the sample of at least 3-fold and up to greater than 10-fold.

3. An assay method as claimed in claim 1 wherein said assay is capable of detecting holo-TC II at a concentration as low as 9 pM.

4. An assay method as claimed in claim 1 wherein said assay is effected performed by an automated process.

5. An assay method as claimed in claim 1 wherein said specific binding ligand is selected from the group comprising a polyclonal or monoclonal antibody, an antibody fragment, a polypeptide, an oligopeptide, a small organic chemical, a specific binder selected from a combinatorial chemistry or phage display library or a specifically binding sequence of DNA or RNA.

6. An assay method as claimed in claim 5 wherein said specific binding ligand is a monoclonal antibody.

7. An assay method as claimed in claim 1 wherein said specific binding ligand exhibits a high degree of

selectivity and specificity towards TC II and exhibits low affinity towards other TC proteins, in either apo or holo form, or any other cobalamin-binding protein.

8. An assay method as claimed in claim 1 wherein said method comprises contacting an immobilised or an immobilisable TC II or holo-TC II binding ligand with the sample under investigation;

separating a ligand-bound fraction from a non-ligand-bound fraction;

dissociating bound cobalamin from the holo-TC II molecules in the bound fraction and determining the concentration of cobalamin released, said dissociation being so affected that the concentration of the released cobalamin is at least 3 times and up to more than 10 times greater than the concentrations of holo-TC II in the initial sample.

9. An assay method as claimed in claim 1 or 8 wherein said cobalamin is released by changing the temperature or the pH of the surrounding medium.

10. An assay method as claimed in claim 1 wherein the different cobalamin forms are converted to the less light sensitive cyanocobalamin by treatment with KCN prior to contacting said sample with a specific binding ligand.

11. An assay method as claimed in claim 1 wherein said free cobalamin is determined by a competition assay performed by contacting an immobilised binding partner for cobalamin with the dissociated cobalamin of the sample in the presence of labelled ligand which competes with the isolated cobalamin for binding to the immobilised binding partners.

12. An assay method as claimed in claim 1 wherein the binding ligands for TC II are immobilised and bind to both holo- and apo-TC II.

13. An assay method as claimed in claim 1 wherein said method comprises contacting a solid support having immobilised thereon a TC II or holo-TC II binding ligand, with the sample under investigation and also with a non-immobilised ligand,

wherein said immobilised ligand is capable of binding to TC II or holo-TC II, to said non-immobilised ligand or to complexes of said TC II or holo-TC II and said non-immobilised ligand, and said non-immobilised ligand is capable of binding to at least one of said immobilised ligand, TC II or holo-TC II and complexes of said immobilised ligand and TC II or holo-TC II;

wherein if said assay method is a sandwich assay, at least one of said ligands is specific for holo-TC II and if said assay is a competition assay said immobilised ligand is specific for holo-TC II and competitors thereof;

whereby the proportion of said immobilised ligand bound by TC II or holo-TC II, by said non-immobilised ligand or by complexes of said non-immobilised ligand and TC II or holo-TC II is dependent on the amount of holo-TC II present in said sample, and,

said non-immobilised ligand is capable of generating a directly or indirectly detectable signal when bound or when unbound;

separating a bound fraction from a non-bound fraction; and

directly or indirectly determining the non-immobilised ligand bound to the immobilised ligand (the bound fraction) or non-bound and in solution (the non-bound fraction);

where the contacting of the sample and said non-immobilised ligand with the solid support may be

performed separately, simultaneously or sequentially, and if performed separately or sequentially, they may be contacted in either order.

14. An assay method as claimed in claim 13 wherein said non-immobilised ligand competes for binding to the immobilised ligand with the holo-TC II, a high level of unbound non-immobilized ligand is indicative of a high concentration of holo-TC II in the sample and a low level of unbound non-immobilized ligand is indicative of a low concentration of holo-TC II in the sample.

15. An assay method as claimed in claim 13 wherein said non-immobilised ligand binds to the TC II or holo-TC II which is bound in turn to the immobilised ligand, such that a high level of bound non-immobilized ligand is indicative of a high concentration of holo-TC II in the sample and a low level of bound non-immobilized ligand is indicative of a low concentration of holo-TC II in the sample.

16. An assay method as claimed in claim 1 wherein a preliminary separation step is carried out using cobalamin or an analogue or fragment thereof which selectively binds the apo-forms of both TC II and haptocorrin (HC), such that the apo forms of the TC II and HC proteins are bound by the cobalamin, analogue or fragment thereof and separated from the holo-TC II and holo-HC complexes.

17. An assay method as claimed in claim 16 wherein said preliminary step is performed and thus in the subsequent analysis of the holo-TC II and holo-HC complexes, the immobilised or non-immobilised ligands may be specific for holo-TC II or TC II or competitors thereof.

18. An assay method as claimed in claim 16 wherein said cobalamin analogue or fragment thereof is tethered to a support by biotin.

19. An assay method as claimed in claim 16 wherein biotinylated cobalamin is added to the sample under analysis in a non-immobilised form where it binds to the apo-forms of TC II and HC, said sample then being contacted with a solid surface having a binding partner for the biotin immobilised thereon, such that a complex of binding partner-biotinylated cobalamin-TC II/HC then forms which can be easily isolated from the sample.

20. An assay method as claimed in claim 19 wherein said biotin binding partner is avidin.

21. An assay method as claimed in claim 16 wherein the holo-TC II pool is determined after separation by contacting the sample with an immobilised TC II ligand which captures the holo-TC II complex leaving the haptocorrin in solution and then contacting the immobilised holo-TC II with a second TC II ligand which is labelled and thus detectable.

22. An assay method as claimed in claim 16 wherein after separation, said holo-TC II molecules compete with labelled non-immobilised ligand for binding to immobilised ligand therefor and the amount of holo-TC II is determined in relation to the amount of labelled non-immobilised ligand bound or not bound to said immobilised ligand.

23. An assay method as claimed in claim 18 wherein the binding of apo TC II and apo HC to cobalamin, analogues or fragments thereof takes place at a site or in such a manner which inhibits subsequent recognition and binding of the immobilised cobalamin bound TC II by the non-

~~immobilised ligand or binding~~ partner for TC II.

24. An assay method as claimed in claim 1 wherein a fraction is separated from the sample which comprises at least a portion of the desired TC II subset of proteins.

25. An assay method as claimed in claim 1 wherein said non-bound fraction is at least 80% ~~, 90% or 95%~~ free of either TC II or holo-TC II.

26. An assay method as claimed in claim 1 further ~~c~~comprising a preliminary separation step in which the sample is contacted with an immobilized or immobilizable specific binding ligand for haptocorrin.

27. An assay method as claimed in claim 1 wherein said TC II binding ligand possesses an affinity constant of at least $10^9 M^{-1}$.

28. An assay method as claimed in claim 27 wherein the affinity constant is greater than $2x10^9 M^{-1}$.

29. An assay method as claimed in claim 28 wherein the affinity constant is greater than $10^{10} M^{-1}$.

30. An assay method as claimed in claim 29 wherein the affinity constant is greater than $10^{11} M^{-1}$.

31. An assay method as claimed in claim 1 wherein the degree of cross reactivity of a holo-TC II or TC II binding ligand with HC is less than 1%.

32. An assay method as claimed in claim 31 wherein the degree of cross-reactivity is between 0.1% and 1%.

33. An assay method as claimed in claim 32 wherein the degree of cross-reactivity is less than 0.1%.

34. An assay method as claimed in claim 1 wherein the binding ligands for TC II or holo-TC II concentrate the ligand by at least 3-fold.

35. An assay method as claimed in claim 34 wherein the binding ligands for TC II or holo-TC II concentrate the ligand by at least 5-fold.

36. An assay method as claimed in claim 35 wherein the binding ligands for TC II or holo-TC II concentrate the ligand by at least 10-fold.

37. An assay method as claimed in claim 1 wherein said sample comprising the holo-TC II complex is contacted with a solid phase to which a labelled ligand recognising the same binding sites on the immobilised ligands as holo-TC II is bound; holo-TC II in said sample competes with said bound labelled ligand for said binding sites such that after equilibration of the system, there is a directly proportional relationship between the amount of labelled ligand displaced from said solid support and detectable in solution and the amount of holo-TC II present in the original sample; said labelled ligand being detected directly or indirectly as the amount of labelled ligand bound or not bound to said solid support as appropriate.

38. An assay method as claimed in claim 1 wherein said holo-TC II containing sample is contacted with a solid support having holo-TC II immobilised thereon and a labelled non-immobilised holo-TC II specific binder, wherein free holo-TC II in the sample and immobilised holo-TC II compete for binding with the labelled non-immobilised ligand; and determination of the labelled ligand bound to the solid phase or remaining in solution allows determination of the holo-TC II concentration.

39. An assay method as claimed in claim 1 wherein said holo-TC II containing sample is contacted with labelled holo-TC II and immobilised ligand therefor; said labelled and non-labelled holo-TC II complexes compete for binding to the immobilised ligand and after equilibrium is reached, the amount of labelled holo-TC II bound to the immobilised ligand is indirectly proportional to the amount of holo-TC II in the sample.

40. An assay method as claimed in claim 1 wherein in a competition assay ,the labelled holo-TC II may be detected directly or indirectly and may be determined as the amount of labelled holo-TC II bound or not bound to the solid support as appropriate.

41. An assay method as claimed in claim 1 wherein in an immunoassay, the labelled holo-TC II may be detected directly or indirectly and may be determined as the amount of labelled holo-TC II bound or not bound to the solid support as appropriate.

Sub D1

42. An assay method as claimed in ~~claim~~ 1 wherein said body sample is ~~selected~~ from the group consisting of ~~comprising~~ seminal fluid, cerebro-spinal fluid, amniotic fluid and ~~or a~~ blood derived samples ~~sample~~.

43. An assay method as claimed in claim 42 wherein said sample is serum or plasma.

Sub D2

44. An assay method as claimed in claim 1 wherein said bound fraction is separated from said unbound fraction by precipitation, centrifugation, filtration or chromatographic methods.

45. An assay method as claimed in claim 1 wherein said detectable ligand is labelled with a signal forming label which may be determined by luminescence,

chemiluminescence, colorimetric assessment, fluorescence, radioactivity or by enzymic activity.

46. An assay method as claimed in claim 1 wherein said immobilised ligand for one or the other components of the apo and/or holo TC II/HC complexes is arranged in a column to which the body fluid containing the TC II cobalamin complex is applied and contacted with the binding ligand(s).

47. An assay method as claimed in ~~claim 1 wherein said~~ the binding ligands used to separate a fraction of a sample are immobilised on a particulate solid phase support.

48. An assay method as claimed in claim 47 wherein said solid support comprises magnetic beads.

~~49~~. A kit for use in a diagnostic assay according to claim 1, comprising:

an immobilized or immobilizable specific binding ligand for TC II or holo-TC II;

a plurality of ~~preferably~~ a holo-TC II ~~solution~~ solutions of known concentration ~~or a set of such solutions having a range of holo-TC II complex concentrations~~;

optionally, a release agent to release cobalamin from holo-TC II; and

~~optionally~~ a labelled ligand.